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Histidine-rich designer peptides of the LAH4 family promote cell delivery of a multitude of cargo

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ABSTRACT

The histidine-rich designer peptides of the LAH4 family exhibit potent antimicrobial, transfection, transduction and cell penetrating properties. They form non-covalent complexes with their cargo, which often carry a negative overall charge at pH 7.4 and include a large range of molecules and structures such as oligonucleotides, including siRNA and DNA, peptides, proteins, nanodots and adeno-associated viruses. These complexes are thought to enter the cells through an endosomal pathway where the acidification of the organelle is essential for efficient endosomal escape. Biophysical measurements indicate that, upon acidification, almost half the peptides are released from DNA cargo, leading to the suggestion of a self-promoted uptake mechanism where the liberated peptides lyse the endosomal membranes. LAH4 derivatives also help in cellular transduction using lentiviruses. Here we compare the DNA transfection activities of LAH4 derivatives, which vary in overall charge and/or the composition in the hydrophobic core region. In addition, LAH4 is shown to mediate the transport of functional β -galactosidase, a large tetrameric protein of about 0.5 MDa, into the cell interior. Interestingly, the LAH1 peptide efficiently imports this protein while it is inefficient during DNA transfection assays.

Abbreviations used:

Aib	α -aminobutyric acid
CMV	cytomegalovirus
DOPC	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPG	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho- <i>rac</i> -(1-glycerol)
DMEM	Dulbecco's modified Eagle medium
DPPA	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphate sodium salt
FCS	fetal calf serum
ITC	isothermal titration calorimetry
MAS	magic angle spinning
NMR	nuclear magnetic resonance
PBS	phosphate buffer saline
PC	1,2-diacyl- <i>sn</i> -glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3- phospho-L-serine
PS	1,2-diacyl- <i>sn</i> -glycero-3- phospho-L-serine
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

INTRODUCTION

Peptides have a great potential in biomedical applications, not only because their building blocks are biodegradable, but also due to their reduced size. In addition, key parameters such as product identification and quality control are possible and these compounds offer the possibility of a reproducible and scalable production process. Lastly, it is possible to (bio)chemically modify the side chains or termini e.g. by phosphorylation or acetylation, and/or introduce non-natural amino acids into the sequence further expanding their range of physico-chemical properties. Peptides also serve as template structures to create mimetics which further extends the range of possible applications [1].

The family of histidine-rich designer peptides of the LAH4 family (Table 1) was initially designed to investigate the interaction contributions that determine the membrane topology of amphipathic helices using cationic antimicrobial peptides as templates [2]. Many variants have been created over the years [3, 4], with members exhibiting potent antibiotic and antiplasmodial action [3], non-covalent nucleic acid complexation and transfection [5, 6], other cell-penetrating activities [7, 8] as well as the ability to enhance *ex vivo* viral transduction, an approach used in clinical gene therapeutic approaches [9, 10].

The core of the LAH4 peptides consists of alanines and leucines where four histidines are interspersed (Table 1). An amphipathic helix forms in membrane environments where all four histidines are localized on one face. Two lysines at each terminus assure good solubility of the peptides in aqueous environments, serve as membrane interfacial anchors and also aid in nucleic acid binding [11]. In membrane environments, where the LAH4 peptides exhibit a high propensity to adopt α -helical conformations, the histidines exhibit pK values between 5.4 and 6.0 and thereby these residues allow one to tune the hydrophobic moment of these sequences by changing the pH [12]. Therefore LAH4 aligns parallel to the surface of phosphatidylcholine bilayers at pH < 6 and adopts a transmembrane orientation when the histidines are uncharged [13]. At intermediate pH, a flexible domain probably facilitates the membrane insertion during the in-plane to transmembrane transition [12]. The midpoint pH of the main conformational transition in solution of a number of LAH4 derivatives was also investigated with even lower values observed [14]. As the transition is reversible, it is possible to evaluate the transfer energy of amino acid side chains from the membrane interface to the membrane interior [15]. Furthermore, dynamic light scattering, CD spectroscopy and fluorescence self-quenching data indicate that, in aqueous buffers, the peptides disperse in the shape of small α -helical aggregates at neutral pH, but exhibit the hydrodynamic radius of extended monomers in acidic solutions

[16, 17]. Following the discovery of LAH4 [2] another peptide with pH-dependent membrane topology and potential biomedical applications has been developed [18]. This sequence encompasses two aspartic acids and adopts the reverse membrane topology, i.e. transmembrane at low pH and associated with the membrane surface at high pH.

Members of the LAH4 family of peptides exhibit membrane pore-formation in model membranes [16] and antimicrobial action at both neutral and, to an even larger extent, at acidic pH [3] against clinical isolates [19]. Importantly, when the helices align parallel to the membrane surface, they associate into mesophase arrangements of high local density [17]. The interactions of the peptides with the bilayer interface result in a decreased order parameter of the phospholipid fatty acyl chains and considerable curvature strain on the membrane [20].

Some of the LAH4 peptides exhibit excellent DNA [21] and siRNA transfection activities with higher efficiencies than that of well-established compounds such as Lipofectamine, DOTAP and polyethylenimine [6]. The simultaneous antimicrobial and DNA transfection activities of these peptides, that are both retained in the complexes with DNA, are of potential interest for multimodal applications [21], for example in hereditary diseases such as cystic fibrosis, where *Pseudomonas aeruginosa* infections are a major complication.

When the peptides and DNA are mixed at pH 7.4, large transfection complexes form that enter the cells via an endosomal pathway [21]. Zeta-sizer measurements indicate that the complexes carry an overall small positive surface charge density [22]. The size of the complexes can be tuned by changing the salt concentration, thus that hydrodynamic diameters in the 100 nm to micrometer range are obtained [22]. It has been shown by ^{13}C MAS solid-state NMR spectroscopy that LAH4 adopts an α -helical conformation in the pure peptide powder as well as in the transfection complex [23]. Solid-state NMR distance measurements show that the positive lysine termini of the peptide, rather than the histidines, interact with the negatively charged phosphates of the DNA [11]. With two lysine side chains at each terminus, the peptide thereby interconnects distant parts of the extended DNA molecule, as well as different DNA strands, thereby leading to the observed condensation of this anionic biopolymer. With the help of biophysical investigations a mechanistic model for the self-promoted uptake and release and the intra-cellular processes that occur during nucleic acid transfection by LAH4 peptides could be established [23] and will be discussed below.

Furthermore, LAH4 sequences have been shown to act as cell penetrating peptides not only for pDNA, siRNA and CpG oligonucleotides [8], but also for quantum dots [7], lentiviruses used in *ex vivo* gene therapeutic approaches [4], adeno associated viruses (AAV, [10]) as well as protein and peptide-based vaccines [8]. In the latter cases the main goal was to expose the

proteins to an endosomal processing thus peptide fragments are presented by MHC-I receptors and this in turn can induce an immune response by CD8⁺ T cells. However, the question remains if this approach can be more general and include proteins that arrive as functional entities in the cytoplasm because this could open new therapeutic perspectives. Indeed, the problem with proteins that target intracellular biological activity such as p53, cytochrome c and many others is that their cytosolic delivery is difficult since most proteins are unable to spontaneously enter mammalian cells. Different protein delivery technologies have been developed [24-27]. Most of them rely on the fusion or conjugation to cationic molecules, as for example cell penetrating peptides (CPP) [28]. Intracellular delivery efficiencies were unfortunately uneven and depend on several parameters.

Here, our previous investigations were extended by focusing on three mechanistic aspects. First, we will focus on the role of the membranes by asking if it is possible to ameliorate the transfection activities by enriching the cellular membrane with acidic phospholipids such as phosphatidylserines. Second, in order to further evaluate the role of electrostatic and hydrophobic contributions a number of peptides are reviewed and presented that vary in their charge composition at pH 7.4 and/or pH 5. Third, we asked whether these peptides are able to deliver a protein into mammalian cells simply after co-incubation of the CPP with the target protein and to keep it functional during the transduction process.

MATERIALS AND METHODS

Materials

The peptides (Table 1) were prepared by solid-phase peptide synthesis using a Millipore 9050 automatic peptide synthesizer and the standard cycles for Fmoc-chemistry with amino acids from Merck-Novabiochem (Fontenay sous Bois, France). The preparations showed one predominant peak, which was purified by reverse phase HPLC (Gilson, Villiers-le-Bel, France) using a preparative C18 column (e.g. Luna, C18-300Å-5µm, Phenomenex, Le Pecq, France) and an acetonitrile/water gradient. The main peak was collected and its identity and purity (> 90%) were checked by analytical HPLC and MALDI mass spectrometry (MALDI-TOF Autoflex, Bruker Daltonics, Bremen, Germany). The purified peptide was lyophilized and stored at -20°C.

CMV-Luc (SMD2-LucDITR plasmid of 7.6 kb) is an expression plasmid encoding the *firefly luciferase* gene under the control of the human cytomegalovirus (CMV) immediate-early promoter. 1,2-diacyl-3-*sn*-glycero-phosphatidyl-L-serine from bovine brain (PS, ≥ 98.0%), 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DOPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate sodium salt (DPPA) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were obtained from Sigma-Aldrich (St. Quentin Fallavier, France).

Cell culture

Dulbecco's modified Eagle medium (DMEM; Thermo Fisher, Illkirch France) was supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% of fetal calf serum (FCS). We used human hepatocarcinoma cells (HepG2 cells) for the protein transduction assays. For the PS enrichment assays, we used the two following cell lines: SV40 transformed human fetal lung fibroblast MRC-5 cells (MRC5-V2); and the murine embryonic fibroblast cell line NIH3T3.

Treatment of cells with phosphatidylserine

Phosphatidyl-L-serine (PS) dissolved in a chloroform/methanol (95:5) mixture was dried in a glass tube and resuspended in phosphate-buffered saline to a final concentration of 5 mM. Liposomes were generated from this solution using a water bath sonicator. The protocol was the same for the PC, PG and the PA based liposomes. Cells plated in 24-well plates were then incubated with increasing concentrations of PS (added to serum containing medium) for 24h. The successful enrichment of cell membranes of 2 cell lines (MRC5-V2 and NIH3T3) with PS was demonstrated using a fluorescently labelled annexin-V (Annexin-V FITC detection kit

from BD Pharmingen, Le Pont de Claix, France). This protein binds tightly and specifically to PS and is commonly used to measure cell-surface PS levels. The efficiency of enrichment was evaluated using flow cytometry by comparing treated vs non-treated cells.

DNA Transfection experiments

Cells were plated in 24-well plates two days before transfection. At day one, the cells were incubated or not with PS for 24h. At day two, the cells were transfected using the following protocol: four micrograms of DNA (CMV-Luc) and the desired amount of peptide were each diluted in 100 μ l of 150 mM NaCl. Peptide and DNA solution were mixed and incubated for 15 min at room temperature. Notably, under these experimental conditions, the size of the complexes is in the micrometer range [22]. Before adding the complexes to the cells, the mixtures were diluted with serum-free medium to a final volume of 1 ml. Experiments were done in duplicate (0.5 ml/well). After about 3h, the transfection medium was replaced with fresh medium containing 10% serum. Cells were harvested 30h after the beginning of the transfection experiment and the luciferase activity was measured as previously described [29].

Preparation of the Peptide/Protein complexes

0.5 μ g of avidin- β -Gal and increasing amounts of LAH derived peptide or polylysine with a degree of polymerization of ≈ 20 (Sigma-Aldrich, St. Quentin Fallavier, France) were each diluted in 50 μ l of calcium free PBS and gently mixed. After 30 min of incubation at room temperature, the mixture was diluted with serum-free medium to a final volume of 0.4 ml.

Protein transduction experiments

240,000 HepG2 cells were plated in 24-well plates one day before the experiment. Before transduction, the cells were rinsed once with PBS. 0.4 ml of serum-free medium containing the complexes were then transferred into each well. After an incubation time of 3h30 at 37°C the medium was removed, cells were washed twice with PBS, fixed and stained with X-Gal. Briefly, cells were washed twice with PBS, fixed in 0.5% glutaraldehyde for 20 min at 4°C, and washed again. Then, the cells were incubated overnight at 37°C with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Finally, the cells were washed again twice with PBS before the transduction efficiency was evaluated.

RESULTS

Enhancement of transfection by phosphatidylserine and phosphatidylglycerol

We previously studied the effect of LAH4 (Table 1) and derivatives on lipid vesicles in the presence of PS using non-perturbing solid-state NMR techniques [5, 29]. Proton decoupled ^{31}P MAS NMR of the phospholipid headgroups revealed an electrostatic interaction between LAH4 and POPS. Furthermore, a close correlation exists between the transfection efficiency of the peptides on cells cultured *in vitro*, with the disruption of POPS chain order as observed with wide line ^2H NMR of chain deuterated POPS. Notably, this data indicates that in mixed PC/PS membranes the cationic peptides selectively interact with the anionic phospholipids. On the other hand, Coil and Miller reported that the enrichment of cell membranes with PS can increase the entry of enveloped viruses [30]. Taking into consideration these results, we asked whether pre-treatment of cultured cells with PS could also increase the transfection efficiency of LAH4.

First, we checked whether by pre-incubation of the cells with phosphatidylserine liposomes (cf. Materials and Methods section) we were able to successfully enrich the cell membranes of two cell lines (NIH3T3 and MRC5-V2) with PS. To this end, we used fluorescently labelled annexin V - a protein that binds specifically to PS and that is commonly used to measure cell-surface PS levels. The results showed that the cell surface content in PS increased in both cell lines (Figure 1) as described previously by Coil and Miller [30].

Next, after 24h of incubation of the cells with PS (or other lipids) a transfection experiment was conducted. As shown in Figure 2A, incubation of cells with PS (from bovine brain) increases in a dose-dependent manner the transfection efficiency. Using the same protocol, DOPC did not impact the efficiency. Next we asked whether other negatively charged lipids can mimic the effects of PS. As shown in Figure 2B, DOPG was also able to increase the activity of LAH4 whilst this was not the case for DPPA. Finally, we found that the effect of PS on the efficiency of the LAH4 peptide could be reproduced on another cell line (Figure 2C).

Evaluation of different LAH4 mutants

The data shown in Figure 2 suggest that after PS or DOPG treatment the increased transfection is due to electrostatic attraction of the positively charged transfection complexes [22] to the negatively charged surface of the outer monolayer of the membrane. This fits well with the experiments showing that deficiency of the cell surface of anionic heparan sulfate proteoglycans (HSPGs) strongly reduces the transfection activity of cationic carriers [31].

Notably, electrostatic interactions between the peptide and the nucleic acids have already been shown to be important for LAH4 transfection [23]. Changing the overall charge of the peptide provides another means to modulate interactions with the cargo, the plasma and the endosomal membranes. In Table 1 the transfection efficiencies of a number of peptide sequences are reviewed and new data are added, where the overall nominal charge at pH 5 and at pH 7.4 are compared to each other. For the Table, the pK of histidines has been assumed 6.0 although in membrane environments somewhat lower values have been determined experimentally [12]. Furthermore, the amino-terminus is considered charged at pH 7.5 and pH 5 although its pK can be relatively close to neutral. Therefore, the Table probably overestimates the number of positive charges, but the general trends are still apparent.

Table 1 shows a comparison of the transfection efficiency of various sequences. The terminal lysines have been shown to be the contact sites between DNA and the transfection peptides and have therefore not been modified here [11]. Notably when additional lysine replace the four histidines in LAK4 transfection activity is also inhibited [21]. With four lysines at the termini at least four histidines are required for good activity [21]. Modifications of the core residues have revealed that efficient endosomal release was correlated to the transition from transmembrane to in-planar alignments in model membranes upon acidification [21, 32]. Therefore, significant transfection activity is retained upon the introduction of two or four phenylalanine residues in the core region (LAH4-L1-F4; LAH4-L1-F2d, Table 1). Similarly rearranging some of the hydrophobic amino acids (LAH4-L1-Opt; LAH4-L1- β ; LAH4 inv-AL) has relatively little effect on transfection efficiency. In contrast, the presence of a proline in the core region [22] or of α -aminobutyric acid residues has large effects on the transfection efficiency (Table 1) when at the same time the latter amino acid increases the antimicrobial activities against a number of bacteria and fungi (unpublished).

Protein transduction assays

Finally we tested the capacity of three different LAH derived peptides, namely LAH1, LAH4 and LAK4 (Table 1), to deliver a protein into a human hepatocytic cell line. Each peptide was mixed at different w/w ratios in PBS with 0.5 μ g of avidin- β -galactosidase. The mixtures were then diluted in serum-free culture medium and added to the cells. A few hours later, the cells were stained. The results show that with 5 μ g of LAH4, an impressive number of HepG2 cells present blue dots while in the absence of LAH4 (avidin- β -Gal alone) or in the non-treated cells, no cells were β -Gal positive (Figure 3). Notably, since the protein is delivered and not the gene,

it is expected that the cells do not present a homogenous staining. To check whether it is only the cationic nature of LAH4 which is required for the protein delivery, we used the peptide LAK4 and a poly-L-lysine with a degree of polymerization of around 20. Interestingly, although there was an increased staining, as compared to β -Gal alone with both carriers, it was still significantly lower than the one obtained with LAH4. One interesting point to mention is that LAH1, while 50-times less efficient than LAH4 in gene delivery experiments, promoted protein transduction with a comparable efficacy than LAH4 (Table 1).

DISCUSSION

The family of LAH4 peptides has excellent capabilities to transfer small molecules such as exon skipping oligonucleotides [33] or very large cargoes such as a 7600 bp plasmid DNA ($MW=5 \times 10^6$) into the cell interior. It has been demonstrated that the peptide/DNA transfection complexes enter via an endosomal pathway [21]. By dissecting the biophysical properties of LAH4 and its complex, a model was established where transfection complexes of 100 nm to several micrometer diameter are formed outside the cells at pH 7.4 [22]. The supramolecular structures are governed by a very high coverage of peptide (about 1 peptide/ 2 bp) [23] thus the negative surface potential of DNA converts into a slightly positive one of the complex [22]. Acidification in the endosome causes the release of a large proportion of LAH4 peptides from the DNA which are then free to interact and probably lyse the membranes [23]. Such efficient endosomal release is probably the reason for the very good transfection activities of LAH4 for both siRNA [6] and DNA [21]. Whereas the first can now reach its cytoplasmic targets, the latter needs to continue its voyage to the nucleus, which could explain why significant efforts to achieve better DNA transfection by sequence variations of LAH4 resulted in only relatively small additional improvements in transfection activities [5]. Notably, the LAH4 peptides have also been shown highly efficient for transferring a variety of cargoes into cells including CpG oligonucleotides [8], proteins (Fig. 3) [8], adeno-associated viruses [10], lentiviruses [4] and quantum dots [7].

Self-association of complexes

In order for the LAH4 peptides to be efficient during transfection, non-covalent complexes are formed with the nucleic acids and other cargo. Therefore, in all cases known to us, the cargo was mixed with the LAH4 peptide and shortly incubated. The non-covalent nature of the complex assures that the information carried by the nucleic acids can be released and becomes easily available at their cytoplasmic or nuclear destination, respectively. A tighter association of all-D-LAH4 with the DNA could be a reason why this peptide is less efficient in transfection assays than the L-enantiomer (Table 1), although it should be noted that the phospholipids also have stereospecific properties. Whereas all-D and all-L antimicrobial peptides have been shown to be equally active [34], the situation may be different for the interactions between DNA, membranes and the transfection peptides investigated here. Alternative approaches have been developed for other cell penetrating peptides where a covalent linkage is cleaved in the reducing environment of the cell interior, but this has so far not been tested for LAH4 [35].

Therefore, the question arises how such a wide variety of compounds can non-covalently interact with the cationic amphipathic LAH4 peptides? For DNA it has been shown previously that electrostatic interactions of the lysines and the phosphates play an important role during complex formation [11, 23]. The association between peptide and DNA results in an overall cationic complex not far from charge neutrality [22, 23], which results in the release of considerable amounts of peptide when the pH drops around the pK of the histidines, thereby making the peptides more positive (Table 1). The peptide association with siRNA and oligonucleotides can be explained in a similar manner.

LAH4 peptides have also been used to transfer proteins and peptides into cells [8] (Figure 2). The pI values of ovalbumin (44 kDa), whose intracellular delivery is reported in reference [8], and avidin- β -galactosidase (530 kDa; [36]) are < 5 when extracted from technical data sheets or by applying a algorithm for such calculations [37], indicating that these proteins are negatively charged at pH 7.4 or 5. The pI of firefly luciferase (61 kDa) around 6.1 is indicative that the protein changes from negative to positive inside the endosome thereby further promoting the release of LAH4. Notably, LAH4 was not only used to deliver the protein directly into the cell [8], but alternatively a plasmid encoding its sequence has been used as a convenient monitoring system for efficient DNA transfection [21]. For the donkey IgG antibody used in reference [8], information about the pI is limited. However, sequence information about exons 1 to 4 of the closely related horse IgG heavy chains subclasses 4 and 7 [38, 39] suggest a pI close to neutral, which corresponds to pI data for other IgGs found in mammalian serum. IgG antibodies typically have a MW around 150 kDa [40]. Consistently, the peptides used in the LAH4-aided immunisation all carried a net negative charge [8].

When the LAH4-mediated increase in transduction of viral particles was investigated, the presence of 100 or 500 mM phosphate considerably reduced the efficiency [10] suggesting that also in this case electrostatic interaction contributions are important for complex formation and cellular import. Indeed negative surface potentials were measured for AAV2 capsids using zeta-sizer measurements [41, 42]. On average, 2700 LAH4 molecules have been found associated with one viral particle during the transduction experiments [10], which corresponds to a more than complete coverage of a sphere of 20 nm diameter (the approximate size of AAVs [43]) with helical peptides. Similarly, an average of 50 peptides has been found associated with each quantum dot (a few nm in diameter) functionalized with PEG-dehydrolipoic acid ligands [7], which thereby offer an amphipathic polar surface for peptide association.

Notably, the avidin- β -galactosidase (530 kDa) presented here is to our knowledge the biggest protein imported so far (Fig. 3) by LAH4 cell penetrating peptides, when at the same

time the 7600 bp DNA plasmid (≈ 5 MDa) [21] or an adeno-associated viral particle [10, 44] represent an order of magnitude larger cargo.

Interactions with the membrane

During the incubation of cargo with the cationic peptides an overall positively charged complex is formed [22, 41], whose interactions with the cellular and cytoplasmic membranes should be favoured by negatively charged surfaces. It has been shown previously that LAH4 preferentially interacts with anionic lipids such as PS in mixed PC/PS membranes [5]. The increased transfection efficiency after incubation of the cells with PS (from bovine brain) or DOPG, but not with DOPC, can be explained by changes in the membrane composition that result in an increased concentration of cationic molecules/complexes next to the now anionic membranes [45]. Notably, 24 h after treatment increased annexin V binding indicates that the PS content of the outer membrane monolayer remains elevated suggesting the stable enrichment of PS in the outer monolayer (Figure 1). The addition of the anionic lipid DPPA did not have an effect on the transfection efficiency. When compared to DOPG and PS from bovine brain, this lipid carries a much smaller head group. It is possible that the resulting cone shaped geometry of the lipid stabilizes the endosomal membrane in the presence of amphipathic peptides [46] thereby counter-acting the more efficient membrane association. Furthermore, the saturated fatty acyl chains of DPPA and the correspondingly high phase transition temperature (75 °C) could be a reason for the absence of transfection enhancement. Similarly, differences in transfection efficiency are observed between cell lines (e.g. [21, 47]) which could involve the composition of the membrane, receptors or other cellular features such as the cell division rate, a process that provides access of cytoplasmic DNA to the nucleus [48].

Endosomal uptake and escape

In the next steps of the transfection process the peptide-cargo complex is taken up by the endosome, where acidification of this organelle is an essential step for efficient release into the cytoplasm [21]. Isothermal titration calorimetry (ITC) indicates that under saturating conditions, and at pH 7.5, one peptide is associated with about 2 base pairs of DNA [23]. Association occurs in the μM range, is driven by electrostatic interactions and reversible. At $\text{pH} < 6$, such as it occurs in the endosomal compartment, the histidine side chains become positively charged and about half of the peptides are released from the transfection complex [23]. Concomitantly, solid-state NMR data indicate that under acidic conditions the packing of

the complexes becomes more relaxed [49]. Notably, changes in the lipid composition of the endosomal membranes potentially also have an effect on the membrane association of the peptides and their topologies (orientation relative to the bilayer surface and degree of self-association) [13], the local pH at the membrane surface [32] and thereby endosomal escape. The liberated peptides are now available to interact with the endosomal membranes at high concentrations to cause lysis of the organelle and efficient release of the cargo. With this mechanism in mind, an oligourea foldamer mimetic of LAH4 has recently been designed and has proven to exhibit similar transfection efficiencies when compared to the native sequences [47].

Thus, to develop full transport activity in the endosomes the peptides need to become significantly more cationic than during the preparation of the complex at pH 7.4 (Table 1). This in turn results in a much increased hydrophobic moment [14, 21] and unbinding from the cargo [23]. Indeed, Table 1 indicates that the most active sequences carry a nominal charge of +5 at pH 7.4 which increases to +9 in the endosome. Changes in activity by modification of the overall charge at either pH 5 or 7.4, rearranging the charges along the sequence of the peptide can be rationalized by this structural requirement (Table 1). Furthermore, when viewed along an Edmundson helical wheel, transfection was optimal when the angle covered by the histidines was between 100° and 120° [5] pointing to the polar angle of the molecule as an important parameter (for definition of polar angle cf. footnote in Table 1). Finally, shortening the core region of the peptide by only two residues much reduces transfection activity [22], which could also contribute to the low efficiency of LAH1, LAH2, LAH3 and LAK4 (Table 1). Notably, many cell penetrating peptides have been designed where endosomal escape remains a limiting factor [50], a step in the transfection process which does not seem to hamper the activities of several of the LAH4 peptides (Table 1). A previously published model based on a wide range of biophysical and biochemical data summarizes the self-promoted uptake pathway of these histidine-rich compounds [23].

When changing the amino acid sequence, any of the steps involved in the transfection process can be affected. Therefore it is quite difficult to understand in detail how and where such mutations change the flow of events. For example, whereas exchanging an Ala and/or Leu with Phe has relatively little effect on the transfection activities, replacing two Leu with Aib in the sequence abolishes all activity (Table 1). Although such a replacement should not have a major impact on overall hydrophobicity [14] the Aib residue is tetra-substituted at its α -carbon. Whereas in crystals, in organic solution or during MD calculations α -helical conformations have been observed for peptides encompassing isolated Aib residues, oligo-Aib peptides prefer

3₁₀ helical conformations [51, 52]. Therefore, in some environments this bulky residue may shift the conformational equilibria in a subtle manner thereby also introducing changes in charge distribution and hydrophobic moment, at least during one of the multiple key steps during the transfection process. Whereas an amphipathic α -helical conformation may still be obtained in a membrane environment [53] other structures concomitant with changes in peptide self-association may be adopted in aqueous solution [14, 32], a notion supported when the CD spectra of LAH4 and LAH4X2 are compared to each other (not shown), or when interacting with the cargo [23]. In this context it is noteworthy that the transfection efficiency of LAH4 and LAH6 variants has been shown to be affected by their propensity to self-associate in solution [14]. Notably, the helix-breaking proline or the introduction of D-amino acids have been shown to also have pronounced effects on the transfection efficiency [22].

In conclusion, a number of LAH4 peptides have been designed with excellent capacities to non-covalently associate and transport a wide variety of cargoes inside cells where a cell promoted mechanism assures endosomal escape. Once the cargoes reach the cytoplasm, individual cargoes have particular requirements, which may be influenced by different intracellular fates and destination endpoints. Therefore, to make these later routes more efficient, improvements in the design of these transport complexes may still be possible.

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Insert Table 1 in landscape format here.

FIGURE CAPTIONS

Figure 1: Cell surface enrichment in PS.

NIH-3T3 and MRC5-V2 cells were incubated for 24h with PS. The successful enrichment of the plasma membranes of the 2 cell lines with PS was demonstrated using a fluorescently labelled annexin-V.

Figure 2: Effect of lipid pre-treatment on the transfection efficiency of LAH4.

NIH-3T3 (A and B) and MRC5-V2 (C) cells were incubated for 24h with various concentrations either PS (bovine brain), DOPG, DPPA or DOPC. The numbers reported for phospholipid contents refer to μM concentrations. Cells were subsequently transfected with 40 μg LAH4/4 μg CMV-Luc complexes (w/w ratio of 10/1) and the luciferase activity was measured 30h post-transfection. The experiments were done in duplicates. * $p < 0.05$ with respect to untreated cells, one way ANOVA with Bonferroni post-hoc test.

Figure 3: Peptide mediated delivery of avidin- β -Gal into HepG2 cells.

Increasing amounts of peptide were mixed with a constant amount of avidin- β -Gal (0.5 $\mu\text{g}/\text{well}$). The complexes were incubated for 3h30 with HepG2 cells plated in 24-well plates the day before. β -Gal activity was revealed after X-Gal staining. Only the best formulation for each compound is shown. Magnification x10 for the photos shown at the top and in the middle; magnification x40 for the photos at the bottom.

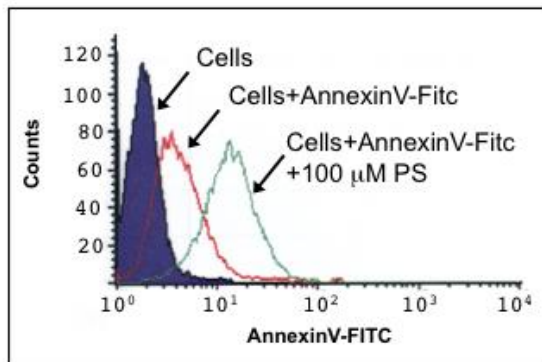
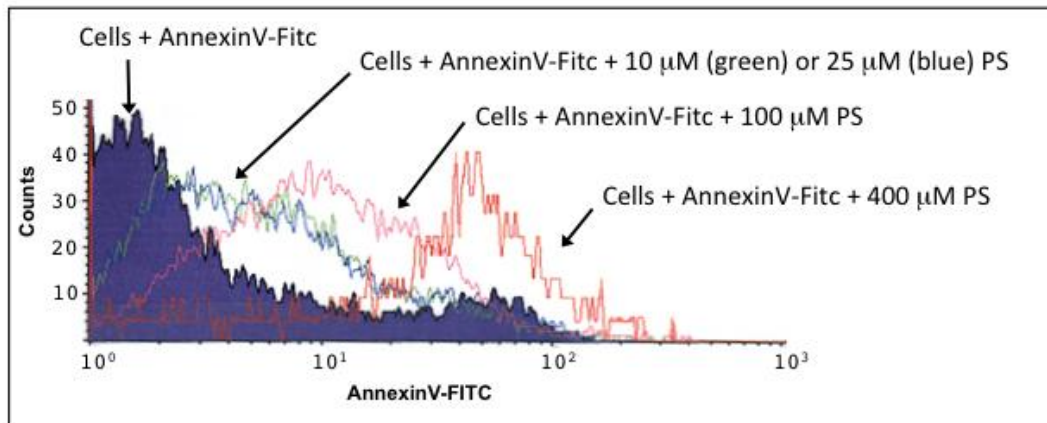


Figure 1

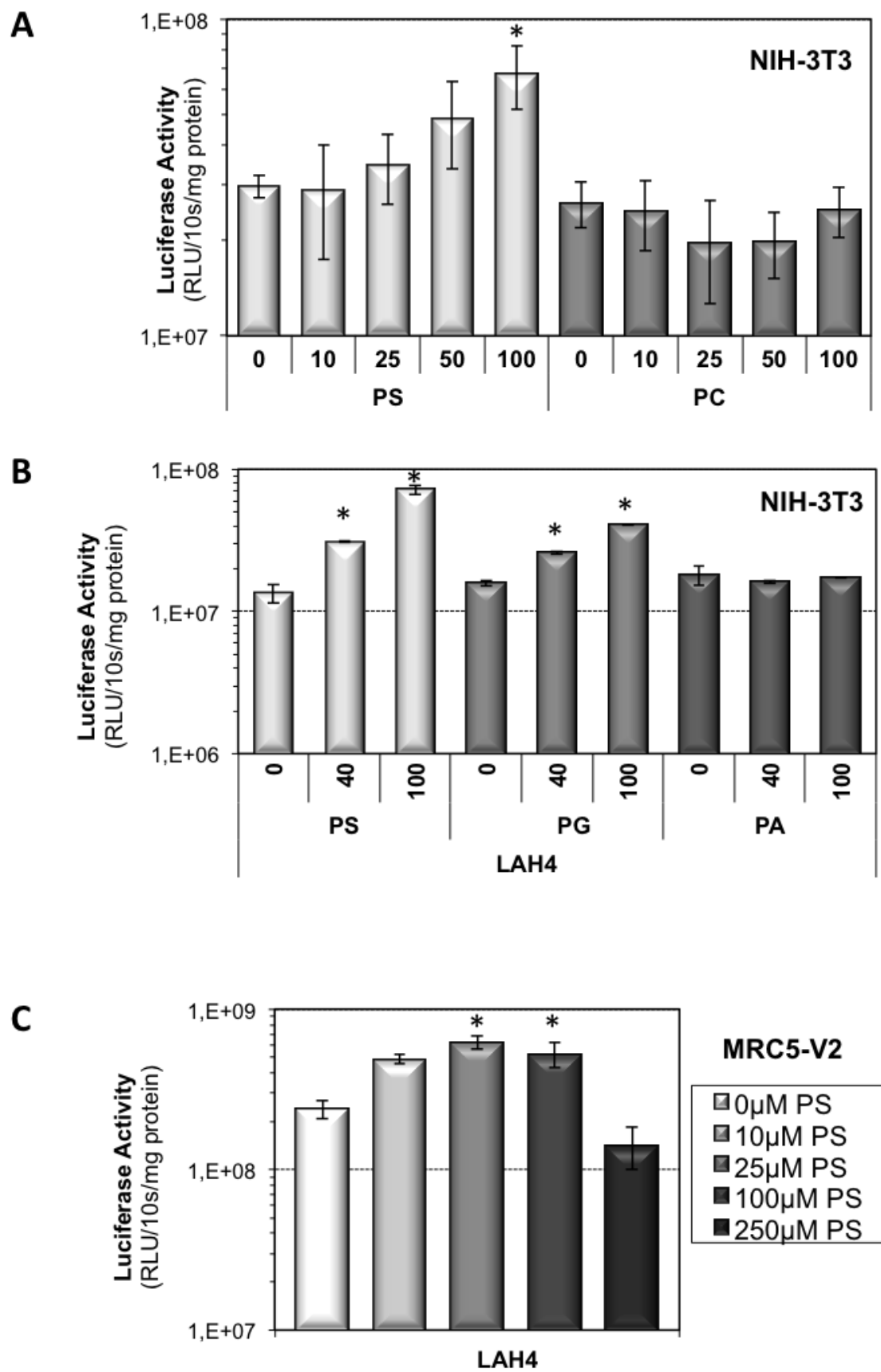


Figure 2

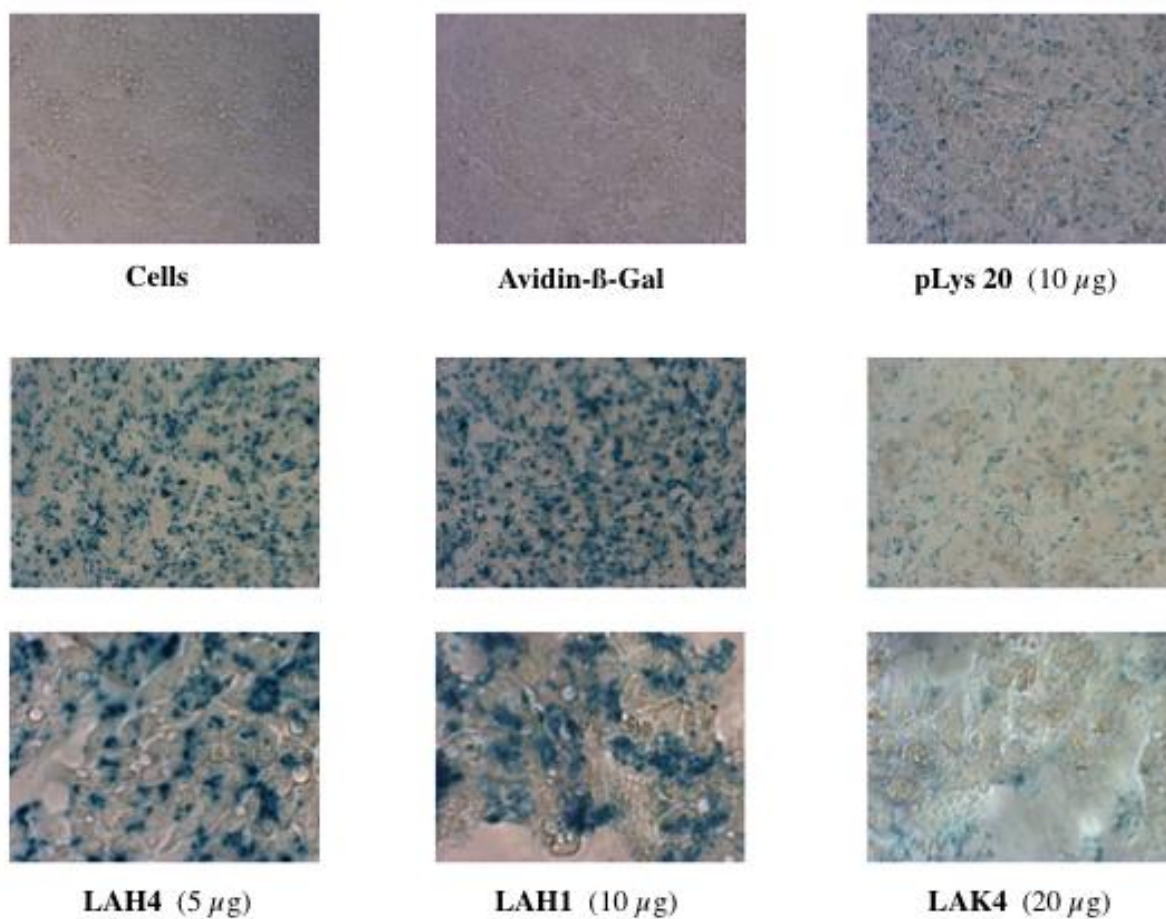


Figure 3

Graphical abstract

